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Effect of challenge dose of plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase producing *Escherichia coli* on time-until-colonization and level of excretion in young broilers

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ABSTRACT

Plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase (ESBL/pAmpC) producing bacteria are present at all levels of the broiler production pyramid. Young birds can be found positive for ESBL/pAmpC-producing *Escherichia coli* shortly after arrival at farm. The aim of this study was to determine the effect of different challenge doses of ESBL/pAmpC-producing *E. coli* on time-until-colonization and the level of excretion in young broilers. One-day-old broilers (specific-pathogen free (SPF) and conventional Ross 308) were housed in isolators and challenged with 0.5 ml ESBL/pAmpC-producing *E. coli* strains of varying doses (10^1–10^5 CFU/ml). Presence and concentration (CFU/gram feces) of ESBL/pAmpC-producing *E. coli* and total *E. coli* were determined longitudinally from cloacal swabs, and in cecal content 72 h after challenge. Higher challenge doses resulted in shorter time-until-colonization. However, even the lowest dose (10^1 CFU/ml) resulted in colonization of the broilers which excreted > 10^6 CFU/gram feces 72 h after inoculation. Conventional broilers were colonized later than SPF broilers, although within 72 h after challenge all broilers were excreting ESBL/pAmpC-producing *E. coli*. A probabilistic model was used to estimate the probability of colonization by initial inoculation or transmission. The higher the dose the higher the probability of excreting ESBL/pAmpC-producing *E. coli* as a result of inoculation. In conclusion, low initial doses of ESBL/pAmpC-producing *E. coli* can result in rapid colonization of a flock. Interventions should thus be aimed to eliminate ESBL/pAmpC-producing bacteria in the environment of the hatchlings and measures focusing at reducing colonization and transmission of ESBL/pAmpC-producing *E. coli* should be applied shortly after hatching.

1. Introduction

Plasmid-mediated extended-spectrum β-lactamase and AmpC β-lactamase (ESBL/pAmpC) producing bacteria are resistant to extended-spectrum cephalosporins (ESC), and are present in humans, animals and the environment (Blaak et al., 2015; Dorado-Garcia et al., 2018). Studies in European countries have revealed that the prevalence of ESBL/pAmpC-producing *Escherichia coli* in broilers is high (Saliu et al., 2017; MARAN, 2019) and ESBL/pAmpC-producing *E. coli* can also be present in chicken at higher levels of the broiler production pyramid such as in (grand)parent stocks (Dierikx et al., 2013). Several field studies have shown that young birds can be positive for ESBL/pAmpC-producing *E. coli* within the first week after arrival at farm (Dierikx et al., 2013; Huijbers et al., 2016; Dame-Korevaar et al., 2017). Transmission of ESBL/pAmpC-producing bacteria occurs throughout the broiler production pyramid via several routes. At every level of the broiler production pyramid young chickens can become colonized as a result of vertical transmission between generations, at hatcheries, horizontal transmission at the farm, between farms and via the environment (Dame-Korevaar et al., 2019). However, the concentrations of ESBL/pAmpC-producing bacteria these birds are exposed to are not known. Therefore, it is important to understand what level of exposure leads to colonization, and at what age birds are colonized by ESBL/pAmpC-producing bacteria. This information is needed to apply successful interventions to reduce transmission within the pyramid. Transmission of ESBL/pAmpC-producing bacteria can occur between subsequent flocks

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(Dame-Korevaar et al., 2019) and therefore colonization of young chickens by ESBL/pAmpC-producing bacteria can be affected by biosecurity measures between production rounds. Raising broilers in hygienic circumstances (Hiroi et al., 2012) and cleaning and disinfecting the floor between production rounds (Mo et al., 2016) are associated with absence and reduced (Odds Ratio 0.1; 95% Confidence Interval (CI) 0.03-0.60) occurrence of ESC-resistant *E. coli*. However, even after intensive cleaning and disinfection of poultry houses, ESC-resistant *E. coli* can still be found in broiler flocks. Thus, persistence of low numbers of resistant bacteria in the farm or in the hatchery environment might lead to colonization of young broilers (Oguttu et al., 2008; Schwaiger et al., 2013; Projahn et al., 2017; Daehre et al., 2018; Projahn et al., 2018; Dierikx et al., 2018). The use of competitive exclusion products has shown to reduce colonization, excretion and transmission in broilers, but not to prevent it (Nuotio et al., 2013; Ceccarelli et al., 2017). However, in those studies broilers were challenged with a high dose of ESBL-producing *E. coli* (0.5 ml of 10^5 – 10^8 CFU/ml), whereas in the field broilers will be most likely exposed to much lower levels of resistant bacteria (Laube et al., 2013, 2014). To evaluate the effectivity of potential measures, the relation between the exposure dose and colonization in groups of young broilers needs to be understood. This knowledge will help to assess interventions against colonization and transmission of ESBL/pAmpC-producing bacteria at all levels of the poultry production pyramid.

The aim of this study was to determine the relationship between challenge dose of ESBL/pAmpC-producing *E. coli* on time-until-colonization and the level of excretion. These outcomes were compared for specific-pathogen free (SPF) and conventional broilers, as well as for two different strains of ESBL/pAmpC-producing *E. coli*. To interpret the results of the time-until-colonization we applied a probabilistic model to estimate the probability of colonization after inoculation, and transmission, as a post-hoc analysis after having observed the data.

2. Materials and methods

We conducted two experiments (Table S1). Experiment I consisted of three replicate studies with SPF broilers inoculated with five doses (10^1 – 10^5). Experiment II consisted of two replicate studies with SPF and conventional broilers inoculated with two doses (10^1 – 10^2).

2.1. Birds and housing conditions

Before the start of the experiment samples were taken from the parent flocks, incubators, hatchers and isolators to confirm the absence of ESBL/pAmpC-producing *E. coli*. In experiment I (May – July 2016), 80 (replicates 1 and 2) and 120 (replicate 3) 18-days incubated Cobb/Hybro/Ross crossbred eggs from a specified pathogen free (SPF) parent flock (Animal Health Service, Deventer, the Netherlands) were transported to the animal facilities (Utrecht University, Utrecht, the Netherlands). Age of the parent stock varied between replicates from 31 to 62 weeks. All eggs were individually disinfected using a tissue with 3% hydrogen peroxide, then placed in egg trays in the hatcher and hatched after 3 days. At day 0 of the experiment, hatchlings were collected, tagged with an individual number, weighed and randomly divided over different isolators, with a maximum of fifteen ($n = 15$) broilers per isolator. The extra chicks were used to prevent differences in group sizes at the moment of inoculation. At day 1, ten ($n = 10$) broilers (not sexed, i.e. consisting of males and females) were selected in each isolator for the remainder of the experiment and were inoculated with a specific dose of the ESBL/pAmpC-producing *E. coli* challenge strain. Broilers with signs of reduced health or development, low hatching weight and, if needed, randomly chosen extra broilers were removed from the isolator and euthanized using cervical dislocation.

In experiment II (September – October 2016), in both replicates 50 SPF eggs were transported to the animal facilities and handled according to the same procedures as in experiment I. In addition, at day 0 of the experiment, 50 just hatched conventional broilers (Ross 308) were transported to the animal facilities, individually tagged, weighed and randomly divided over the isolators. The conventional eggs were disinfected with formaldehyde and eggs were treated in the hatcher with 37% formaldehyde solution. No *in ovo* vaccination or antimicrobials were supplied. Age of the parent stock varied between replicates from 45 to 50 (SPF) and 48–53 (conventional) weeks.

Broilers were housed in negative pressure HEPA isolators. Water and standard mashed broiler diet without any antibiotics or coccidiostatics, radiated with 9 Gy, were available *ad libitum*. On the floor of all isolators were paper linings covered with fine wood shavings. Isolator temperature was gradually decreased from 37 °C at day of hatch until 32 °C at the end of the experiment and a lighting schedule of 23 h light per day was applied. During experiment I, 12 broilers (3 in replicate 1, 7 in replicate 2, and 2 in replicate 3) died or were euthanized before the end of the experiment. In experiment II no broilers died during the experiment.

2.2. E. coli challenge

E. coli strain E39.62, which carries the AmpC gene $bla_{\text{CMY-2}}$ on an IncK plasmid, and E38.27, which carries the ESBL gene $bla_{\text{CTX-M-1}}$ on an IncI1 plasmid, which are representative of common gene-plasmid combinations in *E. coli* isolates from broilers (MARAN, 2019; Ceccarelli et al., 2019), were isolated from healthy broilers in previous studies (Dierikx et al., 2010). Both isolates are resistant to cefotaxime and were used to challenge the broilers in experiments I and II, respectively. MacConkey agar (product no. 212123; Becton Dickinson) was used to culture the *E. coli* challenge strains. Cefotaxime (1 mg/l) used for selective plating throughout the study was obtained from Sigma-Aldrich (Poole, Dorset, UK).

Serial dilutions of the *E. coli* strains were prepared on the day of challenge from fresh culture on heart infusion agar (HIS) with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) supplemented with cefotaxime (1 mg/l), after resuspending into saline solution. Bacterial dilutions were measured with the McFarland reader and retrospective colony counting. For experiment I, all ten broilers per isolator were challenged at day 1 by individual oral inoculation with *E. coli* strain E39.62 using a 1 ml syringe without a needle with 0.5 ml of 10^1 – 10^5 CFU/ml (Table S1) prepared in saline solution (0.85% NaCl). For experiment II, all ten broilers per isolator were challenged at day 1 by individual oral inoculation with 0.5 ml of 10^1 – 10^2 CFU/ml (Table S1) prepared in saline solution (0.85% NaCl) of *E. coli* strain E38.27.

2.3. Cloacal and cecal samples

Individual cloacal samples were collected using sterile dry cotton swabs (MW100, Medical Wire & Equipment, England) from all broilers just before challenge, to confirm the absence of ESBL/pAmpC-producing bacteria. In replicate 1 of experiment I all broilers were sampled with cloacal swabs at t = 3, 6, 9, 12, 15, 20, 24, 28, 32, 48, 52, 56 and 72 h after challenge. In replicates 2 and 3 of experiment I, an additional sample was taken at t = 1.5 h. In both replicates of experiment II broilers were sampled at t = 3, 6, 9, 12, 15, 20, 24, 32, 56 and 72 h after challenge. In all replicates, after the last sampling round at day 4, at 72 h after challenge, broilers were euthanized within the isolator by

cervical dislocation and transported to the post mortem room in individually sealed bags. Post mortem examination was done within 30 min after euthanasia of the broiler. Broilers were weighed, checked for exterior and interior abnormalities, sex was determined, and ceca were collected and stored on ice for further analysis.

2.4. Ethics of experimentation

Broilers were observed daily and the presence of clinical signs, abnormal behavior and mortality was recorded. The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands) under registration number AVD108002015314 and all procedures were done in full compliance with all relevant legislation.

2.5. ESBL/pAmpC-producing E. coli detection

All cloacal samples, except the ones used for quantification of ESBL/pAmpC-producing *E. coli* and total *E. coli* (next paragraph), were enriched in 3 ml Luria Bertani (LB) broth. After overnight incubation at 37 °C, 10 µl were inoculated on MacConkey plates supplemented with 1 mg/l cefotaxime and incubated overnight at 37 °C. Cloacal samples were processed and analyzed individually. *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CMY-2-*E. coli* (EXP I) or CTX-M-1-*E. coli* (EXP II). Colonies inconclusive after visual assessment were typed using MALDI-TOF MS (Bruker Daltonik, Germany).

2.6. ESBL/pAmpC-producing E. coli and total E. coli quantification

Cloacal swabs obtained at t = 9, 32, 56 and 72 h after challenge were weighed before and after sampling to determine the amount of feces collected. The weight of the fecal samples on the cloacal swab specimens ranged from 0.01 to 0.43 g. Each swab was suspended in 3 ml LB broth. At 72 h after challenge, content from one of the two ceca was collected post mortem and 0.1–1.0 gram was used to make a 10% dilution in Phosphate Buffered Saline (PBS). A total of 200 µl of the suspension containing the cloacal feces or cecal content was used to prepare tenfold dilution series (10^{-1}–10^{-5}) in saline solution (0.85% NaCl); 10 µl of each dilution were inoculated on MacConkey plates without and with 1 mg/l cefotaxime, which were incubated overnight at 37 °C. Concentrations of ESBL/pAmpC-producing *E. coli* and total *E. coli* were determined semi-quantitatively (CFU/gram feces), based on the highest dilution showing growth of typical *E. coli* colonies and the weight of the feces on the swabs or the amount of cecal content collected (Ceccarelli et al., 2017). *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CMY-2-*E. coli* (EXP I) or CTX-M-1-*E. coli* (EXP II). Colonies inconclusive after visual assessment were typed using MALDI-TOF MS (Bruker Daltonik, Germany).

The LB broth including the swab was also enriched overnight at 37 °C. If no growth of *E. coli* colonies was observed on the MacConkey plates with cefotaxime (except for samples at t = 9 h of experiment I/replicate 1), 10 µl of the overnight enrichment broth were inoculated on MacConkey plates supplemented with 1 mg/l cefotaxime and incubated overnight at 37 °C. If colonies were detected, the concentration was assumed to be below detection level of the dilution series and the concentration designated as such (Statistical analysis).

2.7. Statistical analysis

First, results of experiment I and experiment II were analyzed separately. Secondly, data of broilers challenged with dose 10^1 or 10^2 CFU/ml from experiment I and experiment II were analyzed together to compare different *E. coli* strains with diverse plasmid/gene combinations. All results were analyzed using R, version 3.4.3. (RStudio Team, 2016), package "survival" (Cox proportional hazard regression), "lme4" (mixed linear regression model) and "bbmle" (maximum log likelihood).

2.7.1. Time-until-colonization

The time-until-colonization was analyzed using Cox proportional hazard regression. Validity of the assumptions of proportional hazards was checked using Schoenfeld residuals and assumptions were met. Colonization of individual broilers was measured by excretion of ESBL/pAmpC-producing *E. coli* and defined as the time point of the first cloacal swab of two consecutive cloacal swabs tested positive for ESBL/pAmpC-producing *E. coli*.

2.7.2. Level of excretion

The effect of challenge dose on the level of excretion was analyzed using a mixed linear regression model including variables *time*, *isolator*, *replicate*, *sex* and *body weight at hatch* and the interaction between *time* and *dose level*. *Animal* was included as random effect to account for repeated measurements for the same broiler. *Body weight at hatch* was included as continuous variable, the others as categorical values.

In addition, in experiment II the variable *type of broiler* (conventional or SPF), and in the analysis of experiment I + II the variable *E. coli strain* was included. The best fitting model was obtained by backward selection, choosing the model with the lowest AIC value. Models with a difference in AIC of 2 or less were considered of equal fit and the most parsimonious model (lowest number of parameters) was chosen.

Broilers negative for ESBL/pAmpC-producing *E. coli* in the dilution series but positive after overnight culturing were included in the analysis with excretion level 1 $\log_{10}$ CFU/ml LB, as the minimum detection level of the semi-quantitative method was 2 $\log_{10}$ CFU/ml LB. Results from the dilution series where no ESBL/pAmpC-producing *E. coli* were counted following a dilution with a high amount of CFU ESBL/pAmpC-producing *E. coli* or the other way around, or results based on negative swab weight (or weight = 0 g) were excluded from the analysis. Moreover, samples negative for ESBL/pAmpC-producing *E. coli* after overnight culturing were excluded since the analysis of excretion levels was based on excreting broilers only. Differences in cecal content ($\log_{10}$ CFU/gram) between the different dose levels and type of broilers were tested using a non-parametric Kruskal-Wallis test.

2.7.3. Probability of inoculation or transmission resulting in colonization

The observed differences in time-until-colonization within isolators showed that colonization of broilers by ESBL/pAmpC-producing *E. coli* can be the result of inoculation leading to colonization or, in case inoculation did not lead to colonization, the result of transmission from other excreting broilers. Post-hoc analysis was performed assuming that the number of ESBL/pAmpC positive broilers as a result of inoculation or as a result of transmission at a point in time has a binomial distribution with

$$1 - \pi = (1 - \pi_{in})(1 - \pi_{tr})^{nprev}$$

and depends on the number of broilers still at risk just before this time point (van den Broek and Heesterbeek, 2007). The probabilities were estimated by maximizing the likelihood function. In this model π is the probability of colonization by ESBL/pAmpC-producing *E. coli* during a

Table 1
Hazard Ratio (HR, 95% CI) of time-until-colonization for experiment I (n = 120), II (n = 78) and I + II (n = 95). In experiment I only SPF broilers were included, challenged with different dose levels of CMY-2-*E. coli*. In experiment II SPF and conventional broilers were included, challenged with different dose levels of CTX-M-1-*E. coli*. Results of experiment I and II were combined in order to compare the different *E. coli* strains.

Experiment	Variable		HR (95% CI)
I	Dose[a]	10^1 (reference)	1
		10^2	3.20 (1.76 - 5.82)
		10^3	12.21 (6.03 - 24.72)
		10^4	14.03 (6.44 - 30.57)
		10^5	25.43 (11.27 - 57.38)
	Replicate	1 (reference)	1
		2	1.00 (0.38 – 2.69)
		3	1.99 (0.89 – 4.44)
	Body weight day 0 (hatch)		0.98 (0.90 – 1.07)
	Body weight day 4		1.01 (0.99 – 1.03)
	Sex	Male (reference)	1
		Female	0.88 (0.57 – 1.35)
II	Dose	10^1 (reference)	1
		10^2	3.94 (2.31 – 6.74)
	Type of broiler	SPF (reference)	1
		Conventional	0.05 (0.01 – 0.22)
	Replicate	1 (reference)	1
		2	0.94 (0.52 – 1.71)
	Body weight day 0 (hatch)		0.94 (0.87 – 1.02)
	Body weight day 4		0.99 (0.95 – 1.02)
	Sex	Male (reference)	1
		Female	1.01 (0.63 – 1.63)
I + II	Dose	10^1 (reference)	1
		10^2	4.80 (2.30 – 10.02)
	Challenge	CMY-2-*E. coli* (reference)	1
		CTX-M-1-*E. coli*	1.22 (0.51 – 2.94)
	Isolator	1 (reference)	1
		2	1.92 (1.01 – 3.64)
		3	0.89 (0.36 – 2.19)
		4	1.03 (0.29 – 3.71)
	Body weight day 0 (hatch)		0.93 (0.86 – 1.00)
	Body weight day 4		1.01 (0.98 – 1.04)
	Sex	Male (reference)	1
		Female	0.93 (0.58 – 1.50)

[a] HR of all dose levels were significantly different ($p < 0.05$), except for dose levels 10^3 and 10^4, and 10^4 and 10^5.

certain time interval, based on π_{in}, which is the probability of colonization because of inoculation, and π_{tr}, which is the probability of colonization by ESBL/pAmpC-producing *E. coli* because of transmission. Probability π_{tr} depends on the total number of broilers being positive at the previous time moment in the same isolator (*nprev*), thus transmission can only occur when at least one broiler in the isolator is excreting ESBL/pAmpC-producing *E. coli*.

The probability of being positive as a result of inoculation (π_{in}) and as a result of transmission (π_{tr}) were estimated using a logistic regression model including time as offset, in which *i* is *dose level*, *j* is *replicate,* and *k* is *type of broiler* (SPF or conventional).

$$\ln\left(\frac{\pi_{in}}{1-\pi_{in}}\right) = \alpha_{in} + \beta_{in_i}X_{in_i} + \beta_{in_j}X_{in_j} + \beta_{in_k}X_{in_k} + \Delta t$$

$$\ln\left(\frac{\pi_{tr}}{1-\pi_{tr}}\right) = \alpha_{tr} + \beta_{tr_i}X_{tr_i} + \beta_{tr_j}X_{tr_j} + \beta_{tr_k}X_{tr_k} + \Delta t$$

For experiment I the model was used without the variable *type of broiler* as only SPF broilers were included, for experiment II the model with all variables was evaluated. The best fitting model was chosen based on the lowest AIC value, if the difference between AIC values was < 2, the model with the least variables was selected as the best model.

3. Results

3.1. Experiment I

3.1.1. The effect of dose on time-until-colonization

All broilers were colonized with CMY-2-*E. coli*, varying between 3 (dose 10^5 CFU/ml) and 24 (dose 10^1 CFU/ml) hours after challenge (Table S2a). Time-until-colonization of CMY-2-*E. coli* depended on the challenge dose, with a hazard ratio for colonization of 3.20 (95% CI 1.76–5.82) for dose 10^2 to 25.43 (95% CI 11.27–57.38) for dose 10^5 (reference dose 10^1, Table 1). Body weight at day 0 and day 4, sex and replicate did not influence the time-until-colonization.

3.1.2. The effect of dose on level of excretion

Excretion levels of CMY-2-*E. coli* and total *E. coli* increased during the experiment (Fig. 1, Table S3). After inoculation (t = 9), excretion levels of CMY-2-*E. coli* increased with the challenge dose, however from t = 32 h onwards no trend with increasing dose was observed. A higher body weight at day of hatch was associated with slightly lower excretion levels of CMY-2-*E. coli*, (-0.07 $\log_{10}$ CFU/gram feces, 95% CI -0.10 – -0.04) (Table S3). All broilers showed cecal content levels of CMY-2-*E. coli* between 7.8–8.3 $\log_{10}$ CFU/gram, with slightly lower levels for dose 10^3 – 10^5 compared to dose 10^1 ($p = 0.02$) (Table S2).

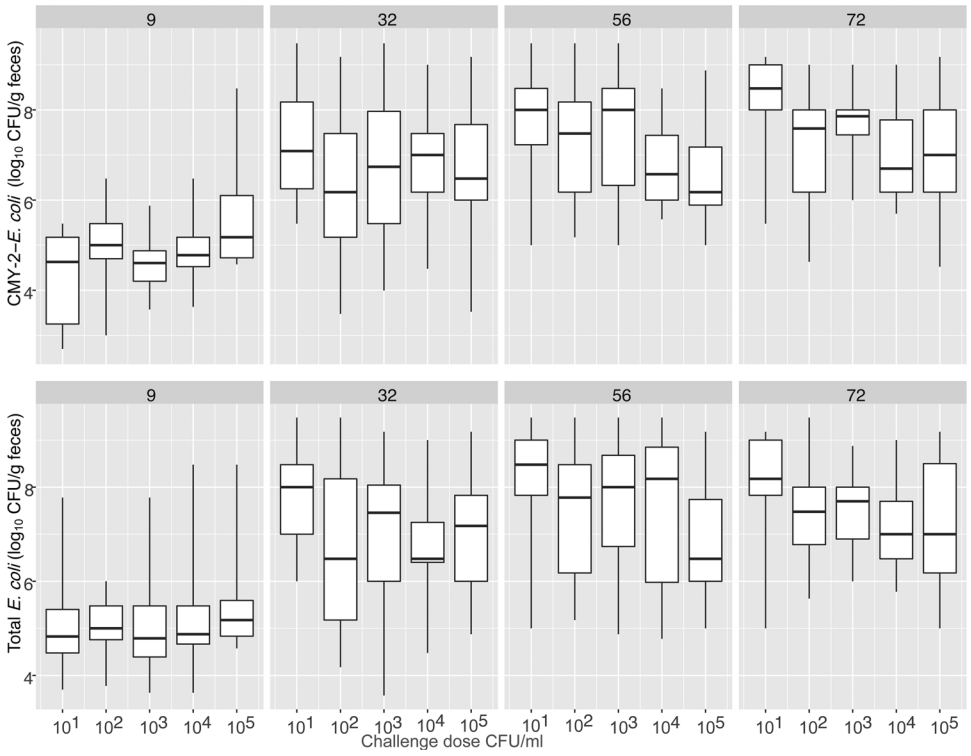

Fig. 1. Experiment I: Excretion levels (log_{10} CFU/g feces) of CMY-2-*E. coli* and total *E. coli* per challenge dose (10^1, 10^2, 10^3, 10^4, 10^5) at 9, 32, 56 and 72 h after inoculation, including broilers with excretion levels above detection limit. The heavy line indicates the median, the box plot extends from the lower to upper quartile, the whiskers indicate the total range of observations.

Table 2
Estimates of probability of becoming colonized per susceptible broiler per hour (95% CI), because of inoculation (π_{in}) or transmission (π_{tr}) for experiment I (n = 159) and experiment II (n = 120), per challenge dose (10^1 – 10^5) and type of broiler (SPF and conventional), analyzed with a model using the maximum likelihood of a binomial distribution with π_{in} and π_{tr}.

Experiment	Variable	Type of broiler	Dose level	Probability positive per broiler per hour (95% CI)
I	π_{in}	SPF	10^1	0.03 (0.01–0.07)
			10^2	0.14 (0.02–0.58)
			10^3	0.34 (0.05–0.81)
			10^4	0.44 (0.07–0.88)
			10^5	0.83 (0.23–0.99)
	π_{tr}	SPF	10^1–10^5	0.04 (0.02–0.06)
II	π_{in}	SPF	10^1	0.05 (0.02–0.10)
			10^2	0.11 (0.02–0.40)
		Conventional	10^1	0.01 (0.001–0.04)
			10^2	0.01 (0.001–0.18)
	π_{tr}	SPF	10^1–10^2	0.05 (0.02–0.08)
		Conventional	10^1–10^2	0.02 (0.005–0.09)

3.1.3. Inoculation and transmission

Colonization of an individual broiler by ESBL/pAmpC-producing *E. coli* in this study was either the result of inoculation or the result of transmission from colonized broilers within the same isolator. Different statistical models to estimate the probability of being positive for CMY-2-*E. coli* as a result of inoculation or transmission were evaluated. In the best fitting model, the probability of being positive as a result of inoculation depended on the dose, while the chance of being positive as a result of transmission was equal for all doses. This model was preferred to a model in which both inoculation and transmission depended on the dose, because of wide confidence intervals around the estimates. The estimated probability for a susceptible broiler to become colonized by CMY-2-*E. coli* as a result of inoculation (Table 2) was 0.03 per hour when challenged with dose 10^1, 0.14 for dose 10^2, 0.34 for dose 10^3, 0.44 for dose 10^4 and 0.83 for dose 10^5. The probability of a susceptible broiler to become colonized as a result of transmission was 0.04, multiplied by the number of excreting broilers in the same isolator per hour (Table 2).

3.2. Experiment II

3.2.1. The effect of dose on time-until-colonization

All broilers were colonized with CTX-M-1-*E. coli*, varying between 56 (dose 10^2 CFU/ml) to 72 (dose 10^1 CFU/ml) hours after challenge (Table S2b). Time-until-colonization depended on the CTX-M-1-*E. coli* dose, with a hazard ratio of 3.94 (95% CI 2.31–6.74) for dose 10^2 (reference dose 10^1, Table 1). For conventional broilers, the hazard rate of colonization by CTX-M-1-*E. coli* was lower than for SPF broilers with a hazard ratio of 0.05 (95% CI 0.01 – 0.22). Body weight at day 0 and day 4, sex and replicate did not influence the time-until-colonization.

3.2.2. The effect of dose on level of excretion

Conventional broilers excreted lower levels of CTX-M-1-*E. coli* compared to SPF broilers (Fig. 2, Table S3). For both conventional and SPF broilers excretion levels increased during the experiment and were higher for broilers receiving challenge dose 10^2, however this difference was only minor (0.66 log_{10} CFU/gram feces, 95% CI 0.23–1.12). Levels in cecal content were also lower in conventional broilers compared to SPF broilers (7.05 versus 8.01 log_{10} CFU/gram cecal content, p = 0.03). *E. coli* was detected in conventional broilers before the moment of inoculation. At t = 9 h after inoculation conventional broilers excreted higher levels of total *E. coli* compared to SPF broilers (Fig. 2). Results of total *E. coli* in isolator 5 of replicate 1 were excluded from the analysis because of a contamination with *Citrobacter freundii* in the isolator, making visual assessment of *E. coli* growth on MacConkey plates not possible.

3.2.3. Inoculation and transmission

Similar to Experiment I, different statistical models were fitted to estimate the probability of being CTX-M-1-*E. coli* positive as a result of inoculation or as the result of transmission from other inoculated broilers within the same isolator. The best fitting model included a probability of being positive because of inoculation, depending on

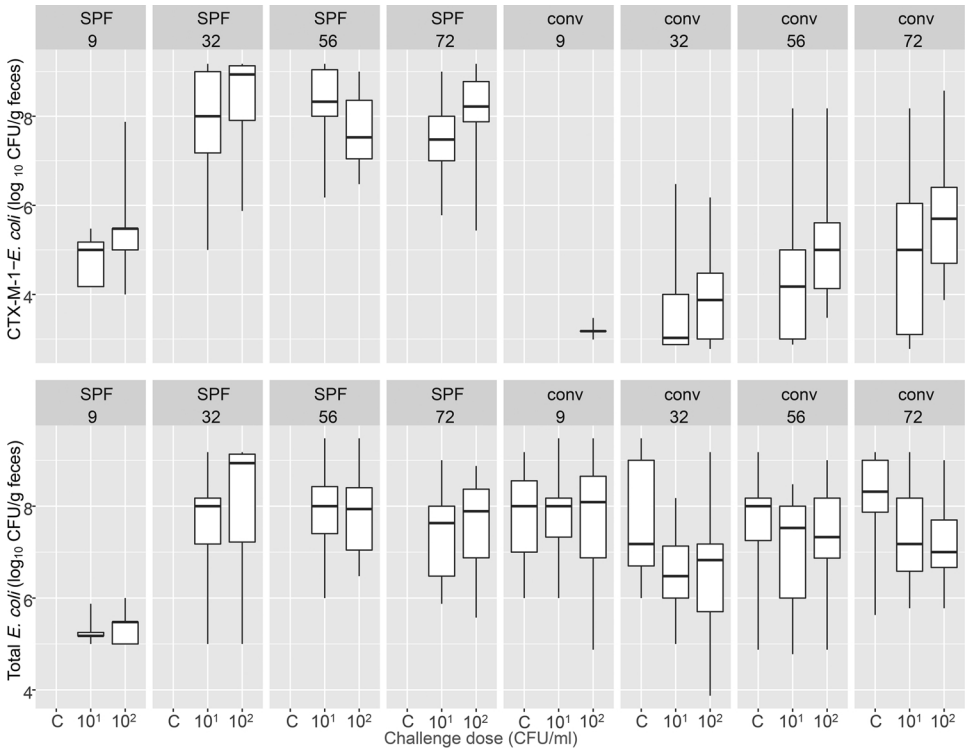

Fig. 2. Experiment II: Excretion levels (log_{10} CFU/g feces) of CTX-M-1-*E. coli* and total *E. coli* per challenge dose (control (C), 10^1 and 10^2) at 9, 32, 56 and 72 h after inoculation, for SPF and conventional (conv) broilers, including broilers with excretion levels above detection limit. The heavy line indicates the median, the box plot extends from the lower to upper quartile, the whiskers indicate the total range of observations.

challenge dose (10^1 or 10^2) and type of broiler (SPF or conventional), and transmission. Transmission depended on type of broiler but was independent of challenge dose. The estimates of the probability of colonization as a result of inoculation were 0.05 (dose 10^1) and 0.11 (dose 10^2) per hour for a susceptible SPF broiler, and 0.01 per hour (doses 10^1 and 10^2) for a susceptible conventional broiler (Table 2). The estimates of the probability of colonization as a result of transmission were independent of dose level and were 0.05 for a susceptible SPF broiler and 0.02 for a susceptible conventional broiler, both multiplied by the number of excreting broilers in the same isolator per hour.

3.3. Experiment I + II

The hazard rate of being colonized did not differ between the two ESBL/pAmpC-producing *E. coli* strains used in experiment I and II for SPF broilers receiving challenge doses 10^1 or 10^2 (Table 1). However, the hazard rate of colonization increased together with the challenge dose with a hazard ratio of 4.80 (95% CI 2.30–10.02) for dose 10^2 versus dose 10^1. Excretion levels of ESBL/pAmpC-producing *E. coli* in both cloacal and cecal samples of SPF broilers did not differ between the two strains (data not shown). Total *E. coli* excretion was slightly lower in SPF broilers challenged with CTX-M-1-*E. coli* (-0.76 log_{10} CFU/gram feces, 95% CI -1.25 – -0.27).

4. Discussion

These experiments have shown that the probability of colonization by ESBL/pAmpC-producing *E. coli* of a single young broiler per hour upon inoculation increases with the challenge dose. Moreover, the time-until-colonization decreases with increasing challenge dose. Eventually, all broilers within the flock became colonized either due to colonization or because of transmission, even after challenge with a dose as low as 10^1 CFU/ml. Furthermore, 72 h after inoculation the level of excretion was not different between the challenge doses. Our results were reproduced for two different *E. coli* strains carrying a different gene-plasmid combination and for both SPF and conventional broilers. Conventional broilers showed a delay in colonization compared to SPF broilers, which might be due to competition by resident *E. coli*, which was detected in conventional broilers before the moment of inoculation. Distinguishing between the rate of colonization by ESBL/pAmpC-producing *E. coli* after challenge as a result of inoculation or transmission shows that presence of only a few ESBL/pAmpC-producing bacteria within a poultry house or hatchery can lead to colonization of some of the broilers, and that subsequently between-broiler transmission will result in a high prevalence of colonized broilers in the flock. This process likely includes, at least in conventional broilers, transmission (via conjugation) of the plasmid present in the inoculum *E. coli* to other *E. coli* strains. In our experimental design, we intentionally decided to follow the resistance phenotype – provided by the ESBL/pAmpC gene encoding plasmid - independently on the *E. coli* strain, which we did not aim to fully characterize. This approach was chosen to reflect the dynamics of ESBL/pAmpC-producing *E. coli* transmission in nature, i.e. the chicken gut (Huijbers et al., 2016; van Hoek et al., 2018), where it is known that horizontal gene transfer occurs and is an integral part of resistance spread in broilers. Yet, to not overlook this biological aspect completely, the use of RAPD PCR as a quick tool to define strain variability was used and the finding of only one RAPD profile in the SPF birds but different profiles in the conventional birds (data not shown) indicated that plasmid transfer to different *E. coli* strains occurred in the conventional birds.

Our method to estimate the probability of colonization by inoculation and transmission may have overestimated the probability of colonization upon inoculation, because a main assumption was that the probability that a bird starts to excrete due to colonization after inoculation remains constant over time during the entire experiment. In reality, the probability that a bird starts excreting as a result of colonization after inoculation, will decline in time, because the initial inoculated bacteria will colonize the bird, or the inoculated bacteria will pass through the gastro-intestinal tract without colonization during the first hours after inoculation. On the other hand, our model did take into account that the probability of a susceptible broiler being colonized because of transmission, will increase in time due to an increase in the number of already colonized broilers excreting ESBL/pAmpC-producing *E. coli*. The model included the number of excreting broilers and did not

take into account the build-up of bacteria, being excreted by the broilers, in the environment, which might have led to underestimation of the infectious pressure. Even though we might have underestimated transmission compared to the probability of colonization by inoculation, the relevance of transmission in dose-effect experiments performed in small groups of broilers is demonstrated, which is in line with the results in earlier experiments with *Campylobacter jejuni* (Line et al., 2008).

Importantly our study provides evidence that the probability of colonization as a result of transmission is independent of the initial dose of ESBL/pAmpC-producing *E. coli* introduced in a flock. Excretion levels higher than the initial challenge doses at 9 h post inoculation, similar excretion levels between the challenge doses quickly after inoculation (32 h post inoculation) in experiment I and only slightly different excretion levels in experiment II do support this idea. Excretion levels of ESBL/pAmpC-producing *E. coli* at the end of the experiment were higher in SPF broilers than in conventional broilers, and were comparable to earlier reported excretion levels in SPF broilers during the first week after challenge (Ceccarelli et al., 2017).

Finding excretion levels similar for all dose levels in experiment I and only slightly different in experiment II indicates that excretion levels do not depend on the initial inoculum, but on processes within the bird. Several bird characteristics might influence excretion levels. In experiment I, broilers with lower hatching weights were excreting slightly higher levels of ESBL/pAmpC-producing *E. coli*. Possibly these hatchlings had limited microbial diversity or abundance, which increased the susceptibility to ESBL/pAmpC-producing *E. coli* colonization, as was suggested earlier for *C. jejuni* (Han et al., 2017). Moreover, other host characteristics such as differences in genetics (reviewed by Kers et al., 2018) between SPF and conventional broilers might affect the microbiome, possibly also in relation to growth performance, and subsequently their response to *E. coli* challenges. In experiment II, conventional broilers showed a delayed time-until-colonization and reduced excretion levels compared to SPF broilers, possibly explained by the microbiota, which included *E. coli*, present before inoculation. The initially present *E. coli* did not carry ESBL/pAmpC, shown by the absence of ESBL/pAmpC-producing *E. coli* in samples at start of the experiment and in the control groups during the experiment. The presence of initial *E. coli* might have reduced susceptibility to the challenge *E. coli*, as previously observed with the supply of competitive exclusion cultures leading to a reduction in colonization (Hofacre et al., 2002; Nuotio et al., 2013; Ceccarelli et al., 2017). The stage of microbiota development and thus the age of broilers might affect the susceptibility to colonization (Jurburg et al., 2019), such as ESBL/pAmpC-producing *E. coli*. Our result show that small amounts of ESBL/AmpC-producing *E. coli* are able to colonize young birds in the early stages of microbiota development.

Inoculation with two different *E. coli* strains with other plasmid and ESBL/pAmpC-gene combinations did not result in differences in time-until-colonization and in excretion levels of ESBL/pAmpC-producing *E. coli* in SPF broilers. Both ESBL/pAmpC-producing *E. coli* strains were obtained from a poultry monitoring program (Dierikx et al., 2010) and were able to colonize and transmit, even in absence of antimicrobials, as observed in earlier studies (Devendec et al., 2011; Fischer et al., 2014; Huijbers et al., 2016; Dame-Korevaar et al., 2017; Ceccarelli et al., 2017). The successful colonization of one of the strains in conventional broilers and both strains in SPF broilers suggest that strains well adapted to broilers are suitable for animal models to study interventions to reduce ESBL/pAmpC-producing bacteria in poultry.

5. Conclusion

The presence of small amounts of bacteria in a hatchery or poultry house could result in colonization of young birds followed by high levels of excretion and transmission within the flock. Interventions, such as hygiene measures, should aim towards eliminating ESBL/pAmpC-producing *E. coli* in the environment of the hatchling, i.e. the hatchery, transport vehicles and the broiler farm. Furthermore, within 72 h of challenge all broilers excreted ESBL/pAmpC-producing *E. coli*, therefore measures focusing at reducing colonization and transmission of ESBL/pAmpC-producing *E. coli,* such as administration of competitive exclusion cultures, should be applied within a very short time frame after hatching.'

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108446.

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